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Allosteric Regulation of Estrogen Receptor Structure, Function, and Coactivator Recruitment by Different Estrogen Response Elements

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Hormone-activated ERs (ERα and ERβ) bind with high affinity to specific DNA sequences, estrogen response elements (EREs), located within the regulatory regions of target genes. Once considered to function solely as receptor tethers, there is an increasing amount of recent evidence to suggest that the sequence of the ERE can influence receptor activity. In this study, we have performed a systematic analysis of the role of different EREs in ER pharmacology. Specifically, by measuring ER activity on the vitellogenin A2, complement 3 gene, pS2, and lactoferrin EREs, we demonstrate that the activities of E2 and xenoestrogen ligands through ERα and ERβ are significantly influenced by the nature of the response element. Using a series of ERα and ERβ interacting peptides that contain the coactivator-binding motif LXXLL, we show that the type of ERE with which the receptor associates regulates the structure of the coactivator pocket on ER. Furthermore, using a novel ELISA developed to measure ER-coactivator interactions revealed that these different conformational states of ERα and ERβ are functionally relevant, as they dictate receptor coactivator binding preference. Together, these results indicate that the DNA response element is a key regulator of receptor structure and biological activity and suggest the ERE sequence influences the recruitment of coactivators to the ER at target gene promoters. We propose that DNA-induced alteration of protein structure and coregulator recruitment may serve as a universal regulatory component for differential gene expression by other nuclear hormone receptors and unrelated transcription factors. (*Molecular Endocrinology* 16: 469–486, 2002)

THE HUMAN ER BELONGS to the nuclear receptor superfamily of ligand-inducible transcription factors, whose members include the receptors for steroids, thyroid hormone, RA, vitamin D, and orphan receptors for which no ligands have yet been identified (1). The classical mechanism of ER-action is similar to that of other nuclear receptors (2). In the absence of hormone, the receptor is sequestered within the nuclei of target cells in an inactive state. The binding of ligand induces an activating conformational change within the ER and promotes homodimerization and high affinity binding to specific DNA response elements (EREs), *cis*-acting enhancers, located within the regulatory regions of target genes. The DNA-bound receptors can then contact the general transcription apparatus either directly or indirectly via cofactor proteins (reviewed in Ref. 3) such as SRC-1 (steroid receptor coactivator-1), GRIP1 (glucocorticoid receptor interacting protein 1), and ACTR (activator of thyroid receptor). ER-coactivator interactions stabilize the formation of a transcription pre-initiation complex and facilitate the remodeling of chromatin at the target gene promoter. Subsequently, depending on the cell and promoter context, the receptor exerts either a positive or negative effect on the expression of downstream target genes.

There are two genetically distinct forms of ER (ERα and ERβ) that display nonidentical expression patterns in target tissues (4–6). *In vitro* studies have revealed that ERα is a more efficient activator of ERE-containing genes than ERβ under most circumstances, and the mechanisms by which these receptors regulate target gene transcription are distinct (5–9). Furthermore, characterization of mice in which either ERα and/or ERβ have been disrupted (αERKO, βERKO, and αβERKO, respectively) has demonstrated that each subtype plays a unique role in ER-action *in vivo* (10–13).

The ligand-dependent transcriptional activity of ERα is mediated by two separate nonacidic activation domains; a constitutive activation function-1 (AF-1) lo-

Abbreviations: ABTS, 2′,2′-Azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid; ACTR, activator of thyroid receptor; BPA, bisphenol A; AF, activation function; C3-ERE, complement 3 gene ERE; 4-CB, 4-hydroxy-2′,3′,4′,5′-tetrachlorobiphenyl; Coum, coumestrol; E2, 17β-estradiol; EBIP, ERβ-interacting peptide; ER, estrogen receptor; ERE, estrogen response element; Gen, genistein; GRE, glucocorticoid response element; GRIP1, glucocorticoid receptor interacting protein 1; GST, glutathione-S-transferase; Lf-ERE, lactoferrin gene ERE; NP, nonylphenol; NR-box, nuclear receptor interaction domain; pS2-ERE, pS2 gene ERE; SRC-1, steroid receptor coactivator-1; 3-TCB, 4-hydroxy-2′,4′,6′-trichlorobiphenyl; TIF-2, transcriptional intermediary protein-2; Vit-ERE, vitellogenin A2 gene ERE; Zear, zearalenone.

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cated within the amino-terminus (A/B domain) and a hormone-dependent AF-2, located in the LBD (14). A functional AF-1 in the human ERβ has not been identified (8, 9); however, both ERα and ERβ contain an AF-2 domain that includes a highly conserved amphipathic α-helix (H12), which is essential for ligand-dependent transcriptional activity and interaction with members of the SRC family of coactivators (3, 15). Specifically, it has been shown that agonist binding to the receptor induces a conformational change that permits the formation of an AF-2 hydrophobic pocket (16, 17). This event enables the receptor to interact with the LXXLL motifs [nuclear receptor interaction domain (NR-box)] contained within the receptor interaction domains of many of the validated coactivators (15, 18). Conversely, the conformational changes induced in ER upon antagonist binding do not permit recruitment of LXXLL motifs (18). Significantly, LXXLL-containing peptides have been identified whose ability to interact with the coactivator-binding pocket of ERα and ERβ is modulated by the nature of the bound agonist (19–22). These data suggest that factors that influence the structure of the AF-2 pocket may regulate cofactor recruitment, and ultimately impact ER pharmacology.

In addition to ligand-specific effects, an additional level of complexity in ER-action was revealed by studies demonstrating that the transcriptional activity of ERα is significantly influenced by the nature of the target promoter (23, 24). The majority of known estrogen responsive genes contain permutations of the consensus vitellogenin ERE (Vit-ERE; GGTCAcagTGACC) within their regulatory regions. Although the affinity of the receptor for the Vit-ERE is higher than for variant sequences, the affinities for many of the imperfect EREs are similar (25, 26). Thus, ER-ERE affinity does not correlate with transcriptional activity and cannot fully explain promoter-specific ER activity observed *in vitro* (27–29). Recently, it was proposed that the response elements recognized by ER and other transcription factors contain information that is interpreted by the DNA-bound protein (30). Likewise, distinct global conformational changes in ERα are apparent on divergent ERE sequences, correlating with differences in ERα-mediated transcriptional activity observed with each element (29, 31, 32). It is becoming increasingly clear that EREs function as allosteric modulators of ER conformation, and that different sequences may alter the receptor in unique manners, perhaps in regions important for transcriptional activation. We hypothesized, therefore, that different response elements might induce distinct allosteric changes in ER AF-2 that modulate binding of coregulator proteins, akin to the way in which different ligands alter receptor structure and function. In the current study, we have investigated whether the conformation of the ERα and ERβ coactivator-binding pocket is influenced by the nature of the response element to which it binds and if this contributes to transcriptional activity and to differential cofactor recruitment by distinct ligand-ER-ERE complexes.

RESULTS

The Transcriptional Activity of the E2 and Xenoestrogen-Bound ERα and ERβ Is Influenced by the ERE Eontext

Transient transfection assays were performed to assess ERα and ERβ transcriptional activity on promoters containing different EREs. HepG2 (human hepatoma) cells were cotransfected with an ERα or ERβ expression plasmid together with a firefly luciferase reporter vector and the pRL-CMV renilla luciferase normalization vector. The reporter vectors contained 1 copy of the vitellogenin, or the nonconsensus pS2, lactoferrin, or complement 3 ERE (Fig. 1A) fused upstream of a minimal TATA promoter and luciferase reporter gene (Vit-ERE-TATA-Luc, pS2-ERE-TATA-Luc, Lf-ERE-TATA-Luc, and C3-ERE-TATA-Luc, respectively). No activity was detected on the 4 ERE reporter vectors in the absence of transfected ER,

A. *Estrogen Response Elements (EREs)*

Gene	ERE Sequence
Vitellogenin A2	GGTCAcagTGACC
pS2	GGTCAcggTGGCC
Lactoferrin	GGTCAaggTAACC
Complement 3	GGTGGcccTGACC

B. *Estrogen Receptor (ER) Ligands*

1. Endogenous ligand: 17β-estradiol
2. Phytoestrogens: genistein, coumestrol, zearalenone
3. Industrial estrogens: bisphenol A
   nonylphenol
   4-hydroxy-2',4',6'-trichlorobiphenyl
   4-hydroxy-2',3',4',5'-tetrachlorobiphenyl

Fig. 1. EREs and ER Ligands Used in Evaluation of ERα and ERβ Transcriptional Activities

A, Sequences of the vitellogenin A2, pS2, lactoferrin, and complement 3 ERE are shown. The ERE half-sites are *capitalized*, and nucleotides that diverge from the consensus vitellogenin A2 ERE half-sites are *underlined*. The 3-bp spacer in each ERE corresponds to the endogenous gene sequence. B, ER ligands used in studies are shown. These include the endogenous ligand E2 and the xenoestrogens (phytoestrogens and industrial estrogens) studied.

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suggesting that no endogenous functional ER was expressed in the HepG2 cells used and that different ERE sequences did not alter the basal (ER-independent) activity of the minimal TATA reporter (data not shown). Using this system, we examined the transcriptional responses of ERα and ERβ with different ER ligands, including E2 and a series of biologically active xenoestrogens (Fig. 1B). The concentration of each ligand used was that yielded maximal ERα and ERβ transcriptional response on each reporter as determined from dose response studies performed with all ligands on each ERE-containing reporter (data not shown). The input quantity of each ER expression vector was equivalent, and was that which yielded maximal activity under all conditions of the assay. Specifically, this concentration was determined by titration of increasing concentrations of ERα or ERβ expression vector and calculating the amount of input receptor that yielded maximal activity on all four reporters with each ligand. This controlled for possible ERE-sequence and ligand-mediated effects on ER-ERE affinity and ERE occupancy in the assay in addition to the potential influence of sequences flanking the response elements. In accompanying control experiments, VP16-ER chimeras were used to measure ER binding to each response element sequence in the presence of different xenoestrogens. Specifically, ERα was fused to the VP16 activation domain to bypass the need for ligand to activate the AFs within the receptor. Thus, when tethered to DNA, the chimeras activate transcription regardless of the agonist/antagonist nature of the bound ligand so that transcription is a quantitative measure of ER-ERE binding. Measuring VP16-ERα activity on each of the 4 ERE-containing reporters revealed that the nature of ligand or ERE sequence did not influence maximal ER-DNA occupancy and relative binding efficacy for each ligand for the 4 EREs were similar (not shown). These observations indicated that variances in reporter gene activity in the transactivation studies would reflect ligand and ERE-mediated effects on ER transcriptional activity rather than differences in ERE site occupancy under each condition.

Using this system, the activity of ERα on different ERE-containing promoters was first examined. It was noted that E2 displayed the greatest efficacy (7.2-fold activation) on the complement 3 gene ERE (C3-ERE) (Fig. 2), whereas no transcriptional response was detected on the pGL2 parent vector containing no ERE sequences (data not shown). Significant activity was also observed on the Vit-ERE, whereas substantially lower response was observed on the Lf and pS2 EREs (65 and 38% compared with the Vit-ERE; Fig. 2 and Table 1). Interestingly, ERα displayed maximal efficacy on the C3-ERE for all of the ligands examined, although the relative activity of ERα on the four response elements was apparent in a ligand-specific manner. For example, the order of efficacy for E2, genistein (Gen), zearalenone (Zear), and 4-hydroxy-2′,4′,6′-trichlorobiphenyl (3-TCB) was C3>Vit>Lf>pS2, respectively, whereas coumestrol (Coum), bisphenol A (BPA), nonylphenol (NP)-activated ERα displayed the following pattern: C3>Vit=Lf>pS2, and for 4-hydroxy-2′,3′,4′,5′-tetrachlorobiphenyl (4-CB), C3>Vit=pS2=Lf (Table 1). These studies reveal that the activity of E2 and xenoestrogen ligands through ERα differ and are dependent on the nature of the target ERE sequence.

The activity of ERβ on different ERE-containing promoters was next examined (Fig. 3). Interestingly, ERβ displayed lower transcriptional activity than ERα for all ligands and EREs examined (compare Figs. 2 and 3). These results are consistent with reports that ERα is a stronger transcriptional activator on ERE-containing promoters (5–9). Notably, however, fold activation of ERβ in most contexts was higher than that of ERα, reflecting the lower basal activity of hERβ noted previously (7–9). As seen for ERα, the activity of ERβ was dependent of the nature of the response element; E2 displayed equivalent efficacy on the Vit and C3 EREs and lower activity on the Lf and pS2 elements (Table 2). The relative activity of ERβ on the 4 EREs was also influenced by ligand (Fig. 3; Table 2). For example, for Gen the order of efficacy was Vit>C3>pS2=Lf, whereas a dramatic change was noted for the 4-CB and Coum-activated ERβ, where Vit>Lf=C3>pS2 and C3>Vit>pS2=Lf, respectively. Taken together, the results of the transactivation studies indicate that EREs function as important regulators of ERα and ERβ-mediated transcription, and there exists a combinatorial effect of ligand and response element on ER activity.

ERα and ERβ Display Unique Preferences for LXXLL Motifs When Bound to Different EREs

One possible mechanism by which different EREs could regulate transcriptional activation by ER is by inducing conformational changes in the receptor at the AF-2 coactivator-binding pocket. These altered conformational states could then have an influence on the cofactor preferences of the receptor-ligand complex. In previous studies, combinatorial phage display technology (33) was used to identify peptides that could probe the structure of the AF-2 binding pocket of ERα and ERβ (19, 22). Most of the peptides identified contained the sequence Leu-X-X-Leu-Leu, a motif shown to constitute the major receptor interaction domain of the p160 class of coactivators (15). In the current study, different phage clones expressing LXXLL peptides were used as conformational probes to test the hypothesis that there are ERE-specific structural differences in the ER AF-2 domain/coactivator-binding pocket. For these analyses, we used peptides that bound to both ERα and ERβ in an agonist-dependent manner (data not shown). A phage ELISA was designed (Fig. 4) in which purified ERα or ERβ was bound to oligonucleotides containing the Vit, pS2, or lactoferrin gene EREs (Lf-EREs) and then incubated with E2 or xenoestrogen ligand. The different ER-ligand-DNA complexes were then probed with predeter-

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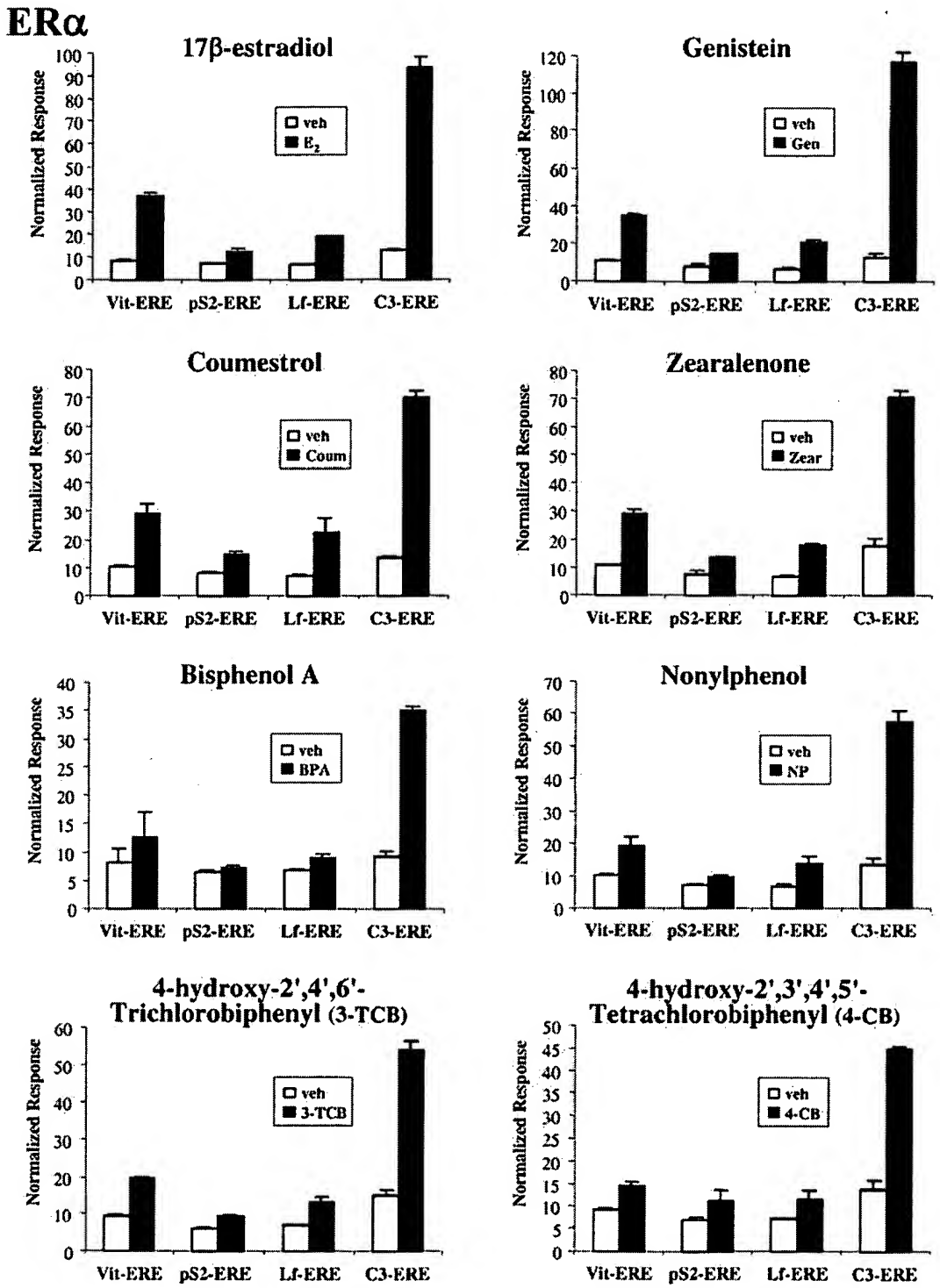

Fig. 2. The Transcriptional Activity of the E2 and Xenoestrogen-Bound ERα Is Influenced by the ERE Context

The transcriptional activity of E2 or xenoestrogen-activated ERα on different ERE-containing promoters was examined. For these experiments, constructs containing the vitellogenin, pS2, lactoferrin, or complement 3 ERE fused upstream of a minimal TATA promoter and luciferase reporter gene were used. HepG2 cells were transiently transfected with an ERα expression vector together with the pRL-CMV normalization plasmid and either the Vit-ERE-TATA-Luc, pS2-ERE-TATA-Luc, Lf-ERE-TATA-Luc or C3-ERE-TATA-Luc reporter plasmids. After transfection, cells were treated with vehicle (veh) or the concentration of each ligand that yielded maximum ERα response on all four reporters as previously determined from dose response studies (10^{-7} M of E2, Gen, Coum, Zear, and 10^{-5} M BPA, NP, 3-TCB, or 4-CB). After 20 h, cells were harvested and dual luciferase assays were performed. Each value was normalized to the internal luciferase control. Each data point is the average of triplicate determinations from a representative experiment, and the average coefficient of variance for each value is less than 10%. Differences in relative activities on the 4 ERE reporters were reproducible in independent experiments for all ligands examined.

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Table 1. Transcriptional Activation of ERα by E2 and Xenoestrogens Is Influenced by the ERE Sequence[a]

ERE	ER Ligand							
	E2	Gen	Coum	Zear	BPA	NP	3-TCB	4-CB
Vitellogenin	100	100	100	100	100	100	100	100
pS2	38[b]	56[b]	76[b]	66[b]	72[b]	69[b]	75[b]	103
Lactoferrin	65[b]	96[b]	108	97[b]	88	99	89[b]	103
Complement 3	165[b]	282[b]	183[b]	150[b]	247[b]	204[b]	171[b]	206[b]

The transcriptional activity of E2 or xenoestrogen-activated ERα on different EREs was examined in HepG2 cells on reporter constructs containing the vitellogenin, pS2, lactoferrin, or complement 3 EREs upstream of a minimal TATA promoter. Cells were treated with vehicle or the concentration yielding a maximal response for each ligand (ranging from 10^{-7} M to 10^{-5} M for E2, Gen, Coum, Zear, BPA, NP, 3-TCB, or 4-CB.

[a] Values represent % of maximal ERα transcriptional activity measured on the Vit-ERE (set at 100%). Activity was calculated by dividing the maximal normalized response of ERα with each ligand on each ERE by the response measured for vehicle treated cells under the same conditions (Fig. 2) in order to correct for differences in basal activity on different ERE-containing promoters.

[b] Indicates significant difference in ERα activity compared with activity measured on the Vit-ERE (error values are included in Fig. 2).

mined saturating concentrations of phage expressing LXXLL-containing peptides (see *Materials and Methods*), and the interaction of different LXXLL motifs with ERα and ERβ was quantitated by ELISA. ERs containing nullifying AF-2 point mutations (9, 24) that have no effect on the ligand-binding characteristics of the receptors, but were shown previously to disrupt LXXLL peptide binding (22), were incapable of interacting with each phage clone in the assay (data not shown). These results indicated that the interaction of peptides used in these studies with the ERs requires a functional AF-2/coactivator-binding pocket, and thus could be used to study the influence of ligand and ERE sequence on the active conformation of this region.

To optimize the amount of ER bound under each assay condition, ER-ERE ELISAs were used to determine the saturating concentration of receptor for each ERE in the assay. This was accomplished by titration of increasing concentrations of ERα or ERβ protein (ranging from 1–10 pmol) on each element and measuring the amount of ER bound with an anti-ER antibody and secondary HRP-conjugated antibody. From these studies, we determined that 4 pmol of ERα or ERβ protein yielded saturation on the Vit, pS2 and Lf-EREs (data not shown); thus, this concentration was used for the ER-phage ELISAs. In additional control experiments we demonstrated that each ligand and peptide did not alter ERα or ERβ binding to any of the 4 EREs by performing ER-ERE ELISAs in the presence of each effector (Fig. 5, A and B, and data not shown). These results confirmed that the amount of ER in each assay condition was constant, indicating variances in peptide binding profiles in the phage ELISAs would reflect changes in the ERα AF-2 domain structure rather than differences in the integrity of the ER-ERE complex in the presence of ligand and peptide.

Using the phage ELISA (Fig. 4), analysis of ERα-phage binding on the Vit-ERE revealed that different LXXLL-containing peptides display distinct patterns of interaction with the E2-bound receptor (Fig. 6A; five representative peptides are shown). This observation agrees with previous reports that LXXLL motifs can differ in their receptor binding preferences (19, 22, 34–36). Analysis of E2-ERα-phage interactions on the Vit, pS2 and Lf-EREs revealed distinct patterns of interactions for all of the peptides examined (Fig. 6A and data not shown). For example, the order of preference on the Vit-ERE was EBIP-95>40>90>82>84, whereas on pS2 the order was EBIP-82>84>95>90>40. Thus, the conformation of the ERα coactivator-binding pocket was influenced by the nature of the response element. ERE-specific patterns of LXXLL peptide-ERα interactions were also apparent in the Coum- and BPA-liganded receptor and with all other xenoestrogens examined (Fig. 6A and data not shown). No binding of phage was detected in the absence of ligand or ER protein, in the presence of purified PR or when GRE (glucocorticoid receptor response element) oligonucleotides were substituted for the ERE oligonucleotides (data not shown).

Analysis of ERβ in the assay also revealed significant ERE-specific effects on the association of different LXXLL motifs with ERβ (Fig. 6B). As seen with ERα, these changes were more apparent when the binding assays were performed in the presence of different ligands. Notably, preferences among the 5 LXXLL peptides were apparent on the Vit, pS2 and C3-EREs and were distinct when examined with the E2, Zear, and NP-liganded ERβ and other xenoestrogens (Fig. 6B and data not shown). Thus, each ERE resulted in a different peptide preference pattern, and in addition, each ligand altered the preference pattern. The two ER subtypes displayed unique peptide binding profiles with different ligands and response elements (Fig. 6, A and B), suggesting that these effectors may influence ERα and ERβ AF-2 conformation in a different manner. Taken together, the results of the phage assays indicate that ERα and ERβ display unique preferences for LXXLL motifs when bound to different ligands and EREs and furthermore, that response

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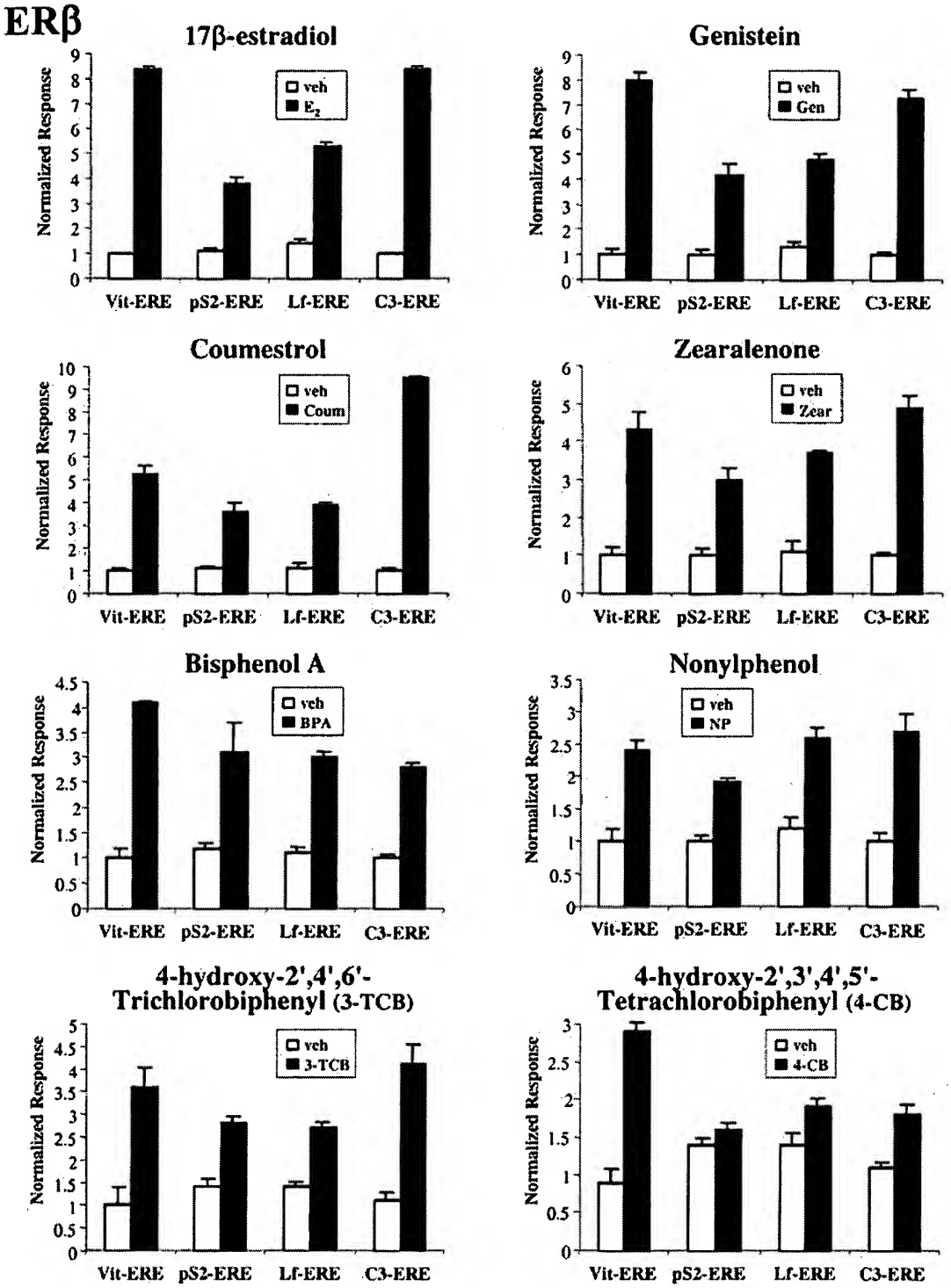

Fig. 3. The Transcriptional Activity of the E2 and Xenoestrogen-Bound ERβ Is Influenced by the ERE Context

The transcriptional activity of E2 or xenoestrogen-activated ERβ on different ERE-containing promoters was examined. For these experiments, constructs containing the vitellogenin, pS2, lactoferrin, or complement 3 ERE fused upstream of a minimal TATA promoter and luciferase reporter gene were used. HepG2 cells were transiently transfected with an ERβ expression vector together with the pRL-CMV normalization plasmid and either the Vit-ERE-TATA-Luc, pS2-ERE-TATA-Luc, Lf-ERE-TATA-Luc, or C3-ERE-TATA-Luc reporter plasmids. After transfection, cells were treated with vehicle (veh) or the concentration of each ligand that yielded maximum ERβ response on all 4 reporters as previously determined from dose response studies (10^{-7} M of E2, Gen, Coum, Zear, and 10^{-5} M BPA, NP, 3-TCB, or 4-CB). After 20 h, cells were harvested and dual luciferase assays were performed. Each value was normalized to the internal luciferase control. Each data point is the average of triplicate determinations from a representative experiment, and the average coefficient of variance for each value is less than 10%. Differences in relative activities on the 4 ERE reporters were reproducible in independent experiments for all ligands examined.

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Table 2. Transcriptional Activation of ERβ by E2 and Xenoestrogens Is Influenced by the ERE Sequence[a]

ERE	ER Ligand							
	E2	Gen	Coum	Zear	BPA	NP	3-TCB	4-CB
Vitellogenin	100	100	100	100	100	100	100	100
pS2	41[b]	53[b]	63[b]	70[b]	63[b]	79[b]	56[b]	35[b]
Lactoferrin	45[b]	46[b]	68[b]	78[b]	82[b]	90	54[b]	42[b]
Complement 3	100	91[b]	183[b]	114	67[b]	113	104	51[b]

The transcriptional activity of E2 or xenoestrogen-activated ERβ on different EREs was examined in HepG2 cells on reporter constructs containing the vitellogenin, pS2, lactoferrin, or complement 3 EREs upstream of a minimal TATA promoter. Cells were treated with vehicle or the concentration yielding a maximal response for each ligand (ranging from 10^{-7} M to 10^{-5} M for E2, Gen, Coum, Zear, BPA, NP, 3-TCB, or 4-CB.

[a] Values represent % of maximal ERβ transcriptional activity measured on the Vit-ERE (set at 100%). Activity was calculated by dividing the maximal normalized response of ERβ with each ligand on each ERE by the response measured for vehicle treated cells under the same conditions (Fig. 3) in order to correct for differences in basal activity on different ERE-containing promoters.

[b] Indicates significant difference in ERβ activity compared with activity measured on the Vit-ERE (error values are included in Fig. 3).

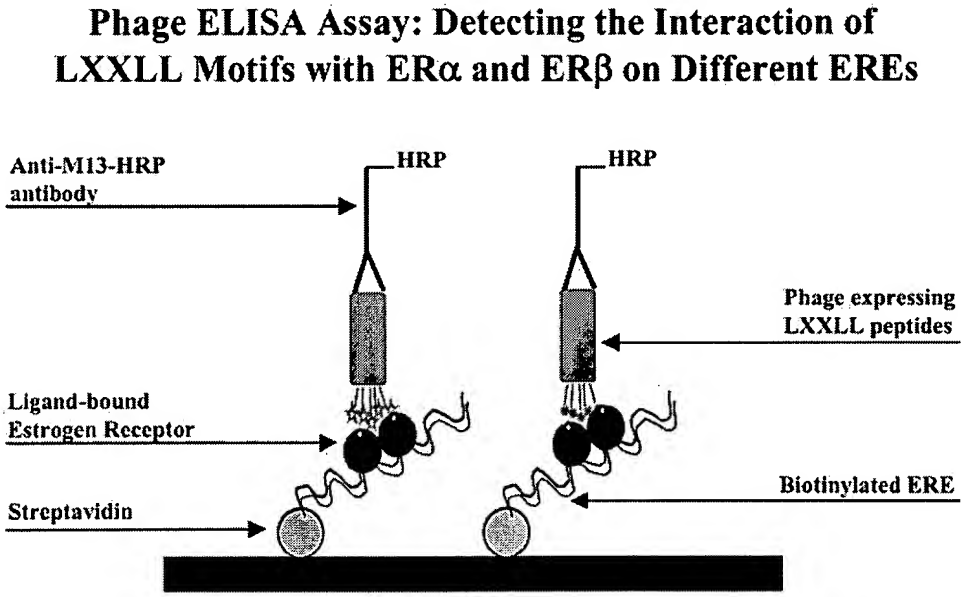

Fig. 4. Development of a Phage ELISA to Probe the Structure of the ERα and ERβ AF-2/Coactivator-Binding Pocket on Different EREs

A phage ELISA was designed to detect ERE-induced conformational changes in the ERα and ERβ AF-2 domains. Ninety-six-well plates were coated with streptavidin, and biotinylated oligonucleotides containing either the vitellogenin, pS2 or lactoferrin gene EREs were added to each well. A saturating concentration of purified ERα or ERβ protein was incubated with the target DNA sequences and 10^{-5} M of different ER ligands. To each well, a saturating concentration (50 μl of a 10^6 pfu titered stock) of individual M13 phage clones expressing peptides containing the coactivator signature binding motif LXXLL were added and incubated with the ER target. The interaction of different LXXLL motifs with ERα and ERβ was quantitated using an anti-M13-HRP (horseradish peroxidase) conjugated antibody and the colorimetic HRP substrate ABTS (2′, 2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid), and by measuring the resulting absorbance for each assay condition at 405 nm.

elements, akin to ligands, function as allosteric modulators of the ERα and ERβ coactivator-binding pocket.

The Specific ERE Sequence Influences the Recruitment of p160 Coactivators to the E2 and Xenoestrogen-Bound ERα and ERβ

The observation that different response elements can alter the interaction of ER with LXXLL motifs suggested that the ERE-specific transcriptional activities observed could reflect ERE-specific influences on coactivator recruitment to the ligand-bound receptor. To test this hypothesis, an ELISA was designed to measure the binding of coactivators with ERα and ERβ when the receptors were associated with different response elements (Fig. 7). Similar to the phage ELISA, a saturating concentration of purified ERα or ERβ was bound to oligonucleotides containing the Vit, pS2, Lf,

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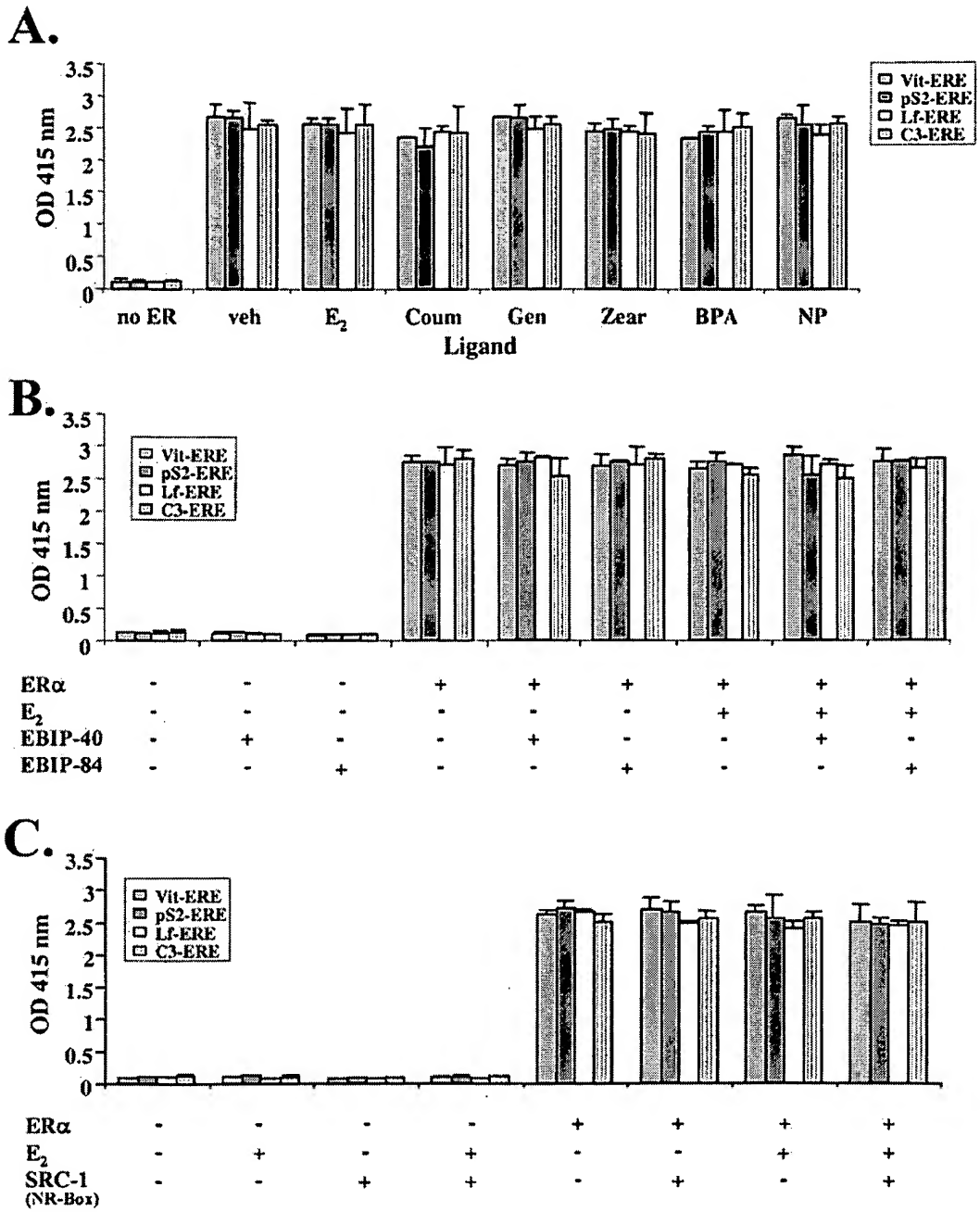

Fig. 5. Validation of Phage and Coactivator ELISAs: Demonstration that Different Ligands, Peptides, and Coactivator Sequences Do Not Disrupt the ER-ERE Complex

A, ER-ERE ELISA control experiments were performed to examine the effect of different ligands on the integrity of the ER-ERE complex. Ninety-six-well plates were coated with streptavidin, and biotinylated oligonucleotides containing either the vitellogenin, pS2, lactoferrin, or complement 3 gene EREs were added to each well. A saturating concentration of purified ERα in PBST or PBST alone (no ER control) was incubated with the target DNA sequences. Vehicle (veh) or 10^{-5} M E2, Coum, Zear, BPA or NP was then added to wells containing the preformed ERα-ERE complexes. The amount of receptor bound under each condition was detected with an anti-ERα antibody and secondary HRP-conjugated antibody and the colorimetic HRP substrate ABTS. The resulting absorbance for each assay condition was measured at 415 nm. Each data point is the average of duplicate determinations from a representative experiment. Very similar results were obtained using other xenoestrogen ligands (listed in Fig. 1B), when ERβ was used in the assay (not shown), and in independent experiments. B, ER-ERE ELISA control experiments were performed to examine the effect of different LXXLL-containing peptides on the integrity of the ER-ERE complex. ELISA plates containing ERα-ERE complexes were prepared as described above. M13 phage clones (50 μl of 10^6 pfu titered stocks) expressing LXXLL peptides (EBIP-40, EBIP-84) were added to individual wells and incubated with the ERα-ERE target in the presence of vehicle or 10^{-5} M E2. The amount of ERα bound under each condition was detected as described above. Each data point is the average of duplicate determinations from a representative experiment. Very similar results were obtained using other peptides, when ERβ was used in the assay (not shown), and in independent experiments. *Amino acid sequences of peptides*: EBIP-40:

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or C3-EREs and incubated with E2 or xenoestrogen ligand. The different ER-ligand-DNA complexes were probed with predetermined saturating quantities of different glutathione S-transferase (GST)-coactivator fusion proteins (see *Materials and Methods*). These included the NR-box (three central LXXLL motifs and flanking sequences) from the p160 coactivators SRC-1, GRIP1, or ACTR, all known to bind ER through these regions (37–39). The interaction of SRC-1, GRIP1, or ACTR NR-box sequences with ERα and ERβ was quantitated using an anti-GST-HRP antibody and colorimetric detection system, and the degree of coactivator interaction on the 4 ERE sequences was compared. No significant interaction was detected in absence of ER, in the presence of PR or GST alone, or when GRE oligonucleotides were substituted for ERE oligonucleotides (data not shown).

To optimize the amount of ER bound under each assay condition, saturating concentrations of receptor for each ERE were used in the assay (4 pmol of ERα or ERβ on the Vit-, pS2-, and Lf-EREs, 8 pmol on the C3-ERE), as determined by ER-ERE ELISAs (see Fig. 5 text). Additional ELISA experiments revealed that SRC-1, GRIP1, ACTR, or different ligands did not alter ERα or ERβ binding to any of the 4 EREs (Fig. 5, A and C, and data not shown). These results confirmed that the amount of ER in each assay was constant, indicating variations in cofactor binding profiles in the coactivator ELISAs would reflect ERE- and ligand-specific influences rather than differences in the number of ER-ERE complexes present under each condition.

Using the coactivator ELISA (Fig. 7), it was determined that the interaction of the E2- or xenoestrogen-liganded ERα with SRC-1 (NR-box) was significantly influenced by the ERE sequence used (Fig. 8A). For most of the ligands examined, the interaction was highest on the C3- and Vit-EREs and significantly lower on the pS2- and Lf-EREs. These results correlate with the transactivation data, where the transcriptional activity of ERα was maximal on the C3 element for all ligands and next highest on the Vit-ERE for most of the ligands studied (Table 1). Furthermore, the relatively low levels of transcriptional activity observed on the pS2 gene ERE (pS2-ERE) were mirrored by the relatively weak interaction of ERα and SRC-1 on this element (Fig. 8A). Quantitation of ERα binding to GRIP1 and ACTR NR box sequences also revealed ligand and ERE-specific patterns of interaction (Fig. 8, B and C). Compared with SRC-1, however, GRIP binding correlated less well with the transactivation data, as the relative levels of GRIP interaction on the pS2 and C3 EREs had no relation to ERα activity on these elements (Fig. 8B; Table 1). The overall efficacy of ACTR binding was reduced compared with that observed with SRC-1 and GRIP1 (Fig. 8). However, ACTR binding mirrored ERα transcriptional response on each ERE for the E2, Zear, and BPA ligands (Fig. 8C; Table 1). Under most circumstances, unliganded ERα displayed basal levels of interaction with all three coactivators, (Fig. 8, A–C), correlating with previous observations that the human ERα displays significant ligand-independent transcriptional activity on ERE-containing promoters (data not shown). Notably, addition of the ER inverse agonist ICI 182,780 completely antagonized coactivator binding by unliganded ERα to the levels displayed by the no ER control (data not shown). Overall, the results from the ERα-coactivator binding studies indicate that the nature of the ligand and response element influences the interaction of ERα with p160 coactivators, and furthermore, there exists a correlation between the magnitude of SRC-1 and ACTR binding and levels of ERα transactivation on different EREs.

Analysis of ERβ in the assay also revealed significant ligand and ERE-specific influences on the binding of p160 coactivators to ERβ (Fig. 9). In correlation with the transactivation data, SRC-1 (NR box) displayed the highest levels of interaction with ERβ on the Vit- and C3-EREs when the receptor was occupied by E2, Gen, Zear, or 3-TCB (Table 2 and Fig. 9A). Furthermore, for the E2, Coum, Zear, BPA, and 3-TCB ligands, the relative levels of ERβ-SRC-1 association on the 4 EREs directly paralleled the transactivation profiles seen on the promoters containing these elements. As seen for SRC-1, the relative levels of ERβ-GRIP1 (NR-box) interaction on the 4 EREs mirrored the transactivation data for most of the ligands examined (Table 2 and Fig. 9B). Interestingly, when the least active compounds on ERβ (Fig. 3; Zear, BPA, NP, 3-TCB, and 4-CB) were used in the assay, the receptor displayed a weaker association with SRC-1 and GRIP1 compared with that viewed with E2, Gen, and Coum (Fig. 9, A and B). The most surprising results in the ERβ studies, however, were observed with ACTR (NR-box), where relatively low levels of interaction were detected that were less sensitive to the ligand and ERE context

GRDDFPL**LISLL**KDGALSL; EBIP-84: NGWHSPL**LRELL**KTQRSSV. C, ER-ERE ELISA control experiments were performed to examine the effect of coactivator NR-box sequences on the integrity of the ER-ERE complex. ELISA plates containing ERα-ERE complexes were prepared as described above. A saturating concentration of purified GST-SRC-1 NR-box (2.5 μg) was added to individual wells and incubated with the ER target. The assays were performed in the presence of vehicle (veh) or 10^{-5} M E2. The amount of ERα bound was detected with an anti-ER antibody, a secondary HRP-conjugated antibody, and the colorimetic HRP substrate ABTS. The resulting absorbance for each assay condition was measured at 415 nm. Each data point is the average of duplicate determinations from a representative experiment, and reproducible trends were observed in independent experiments. Very similar results were also obtained using the GRIP1 and ACTR NR-box fusion proteins, different ligands, and when the experiments were repeated with ERβ (not shown).

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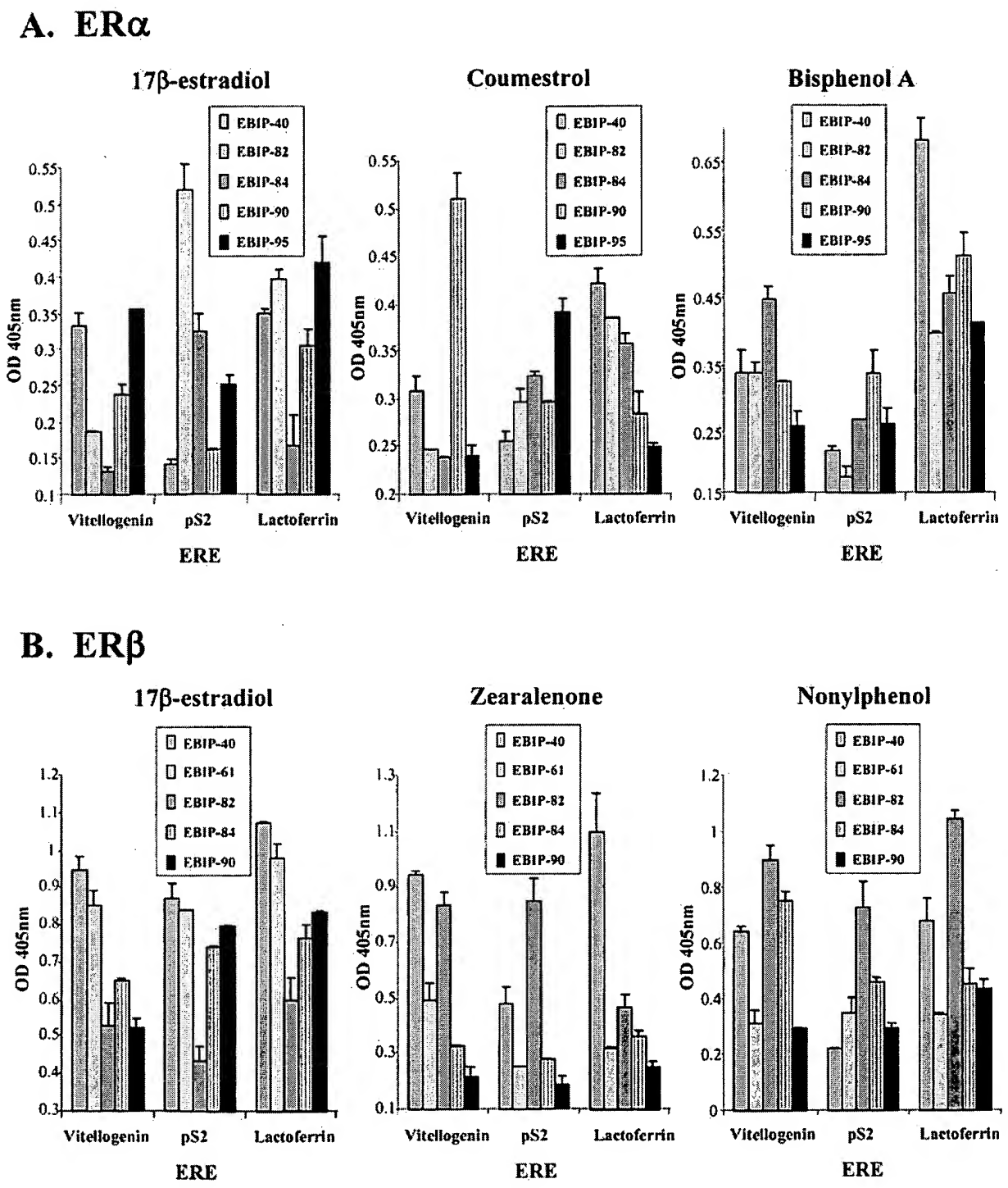

Fig. 6. ERα and ERβ Display Unique Preferences for LXXLL Motifs when Bound to Different EREs

Phage ELISAs were used to detect ERE-induced conformational changes in the ERα and ERα AF-2/coactivator-binding pocket. A, Phage ELISAs were used to measure the binding of phage expressing LXXLL peptides *(EBIP-40, EBIP-82, EBIP-84, EBIP-90, EBIP-95)* to ERα in the presence of 10^{-5} M E2, coumestrol, or bisphenol A. Ninety-six-well plates were coated with streptavidin, and biotinylated oligonucleotides containing either the vitellogenin, pS2, or lactoferrin gene EREs were added to each well. A saturating concentration of purified ERα protein was incubated with the target DNA sequences and 10^{-5} M of different ER ligands. To each well, a saturating concentration (50 μl of a 10^6 pfu titered stock) of individual M13 phage clones expressing peptides containing the coactivator signature binding motif LXXLL were added and incubated with the ER target. The interaction of different LXXLL motifs with ERα was quantitated using an anti-M13-HRP (horseradish peroxidase) conjugated antibody and the colorimetic HRP substrate ABTS (2′, 2′-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid), and by measuring the resulting absorbance for each assay condition at 405 nm. Error values indicate variation among replicate samples for each condition measured in a representative experiment. The results from independent experiments were very similar. B, ELISAs were used to measure the

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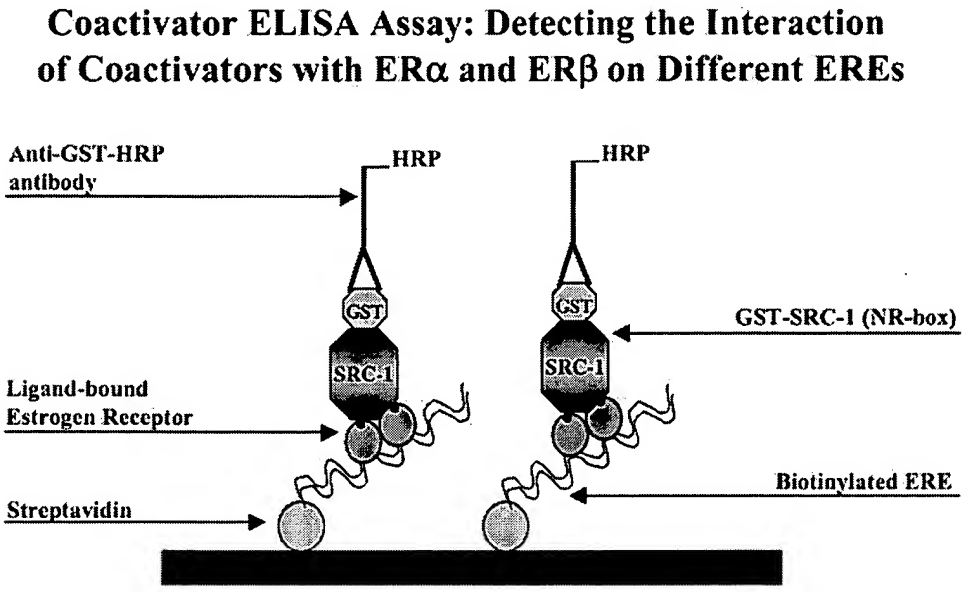

Fig. 7. ELISA Designed to Study the Influence of Different ERE Sequences on Coactivator Recruitment by E2 or Xenoestrogen-Bound ERα and ERβ

Ninety-six-well plates were coated with streptavidin, and biotinylated oligonucleotides containing either the vitellogenin, pS2, lactoferrin, or complement 3 gene EREs were added to each well. Saturating concentrations of purified ERα or ERβ protein (4 pmol for the Vit-, pS2-, and Lf-EREs, 8 pmol for the C3-ERE) were incubated with the target DNA sequences and 10^{-5} M of different ER ligands. To each well, a saturating concentration (2.5 μg) of GST-SRC-1 (NR-box), GST-GRIP1 (NR-box), or GST-ACTR (NR-box) fusion protein, containing the nuclear receptor interaction domain of each coactivator (3 LXXLL motifs), was added and incubated with the ER target. The interaction of different LXXLL motifs with ERα and ERβ was quantitated using an anti-GST-HRP conjugated antibody and the colorimetic HRP substrate ABTS, and by measuring the resulting absorbance for each assay condition at 415 nm. Shown is an illustration of the assay designed to measure ER-SRC-1 (NR-box) interactions.

(Fig. 9C). While ACTR also displayed a lower efficacy in binding ERα compared with SRC-1 and GRIP1 (Fig. 8), ERE specificity was more apparent, suggesting that different response elements communicate with ERα and ERβ in a distinct manner (see *Discussion*). Unliganded ERβ displayed higher basal interaction relative to maximum with all three coactivators compared with ERα (Figs. 8 and 9), as observed previously *in vitro* and in mammalian cells (Hall, J. M., and D. P. McDonnell, unpublished observations). However, as seen for ERα, ligand-independent cofactor binding was completely antagonized by the addition of ICI 182,780 (data not shown). Taken together, the ERα- and ERβ-coactivator binding studies indicate that the ERE is an important regulator of cofactor recruitment by the liganded receptors, and there exists a correlation between ER-coactivator association and the relative levels of ER-mediated transactivation at different target gene promoters.

DISCUSSION

Key Regulatory Role of the ERE Sequence in ER Structure and Function

The tripartite model of steroid hormone action predicts that the transcriptional activity of ER and other nuclear hormone receptors is influenced by three classes of binding partners: ligand, receptor-associated proteins, and the target DNA response element (40). Clearly, a regulatory role for ligand and cofactor association in ER transactivation has been well defined (3, 41–43). Recently, it has been postulated that specific DNA response elements function as allosteric ligands for nuclear receptors, altering their structure and function (30). In the current study, we have demonstrated a regulatory role for EREs in mediating the transcriptional activities of ERα and ERβ. Using a sensitive phage ELISA, ERE-specific preferences for LXXLL mo-

binding of phage expressing LXXLL peptides *(EBIP-40, EBIP-61, EBIP-82, EBIP-84, EBIP-90)* to ERβ in the presence of 10^{-5} M E2, zearalenone, or nonylphenol according to the methods described above. Error values indicate variation among replicate samples for each condition measured in a representative experiment. The results from independent experiments were very similar. *Amino acid sequences of peptides*: EBIP-40: GRDDFPL**LISLL**KDGALSL; EBIP-61: GGTQDGY**LWSLL**TGMPEVS; EBIP-82: YGLKMSL**LESLL**-REDISTV; EBIP-84: NGWHSPL**LRELL**KTQRSSV; EBIP-90: FEQPGGL**LLLLL**QDGAPMN; EBIP-95: LVERESM**LVKLL**RAENPEL.

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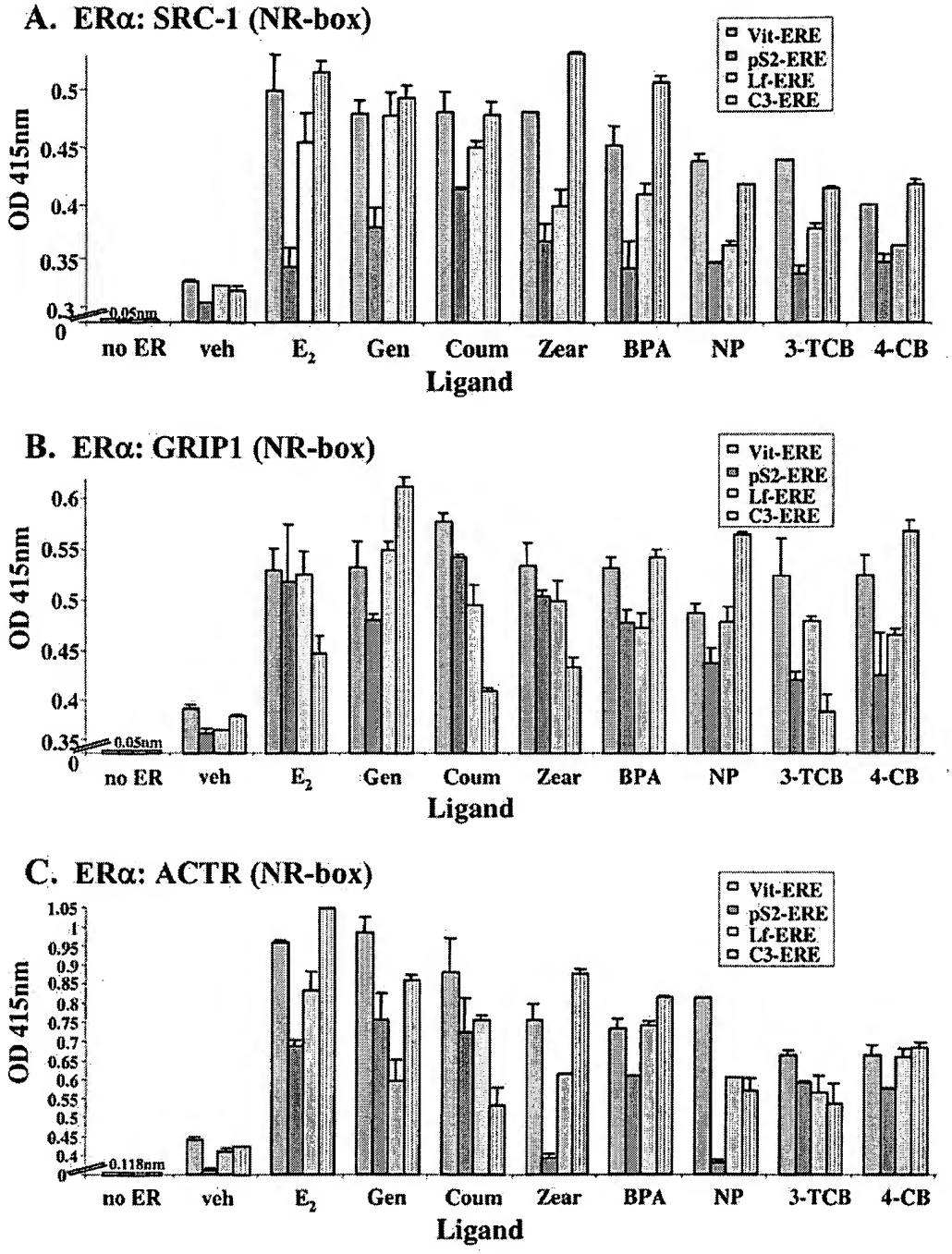

Fig. 8. The Specific ERE Sequence Influences the Recruitment of p160 Coactivators to the E2 and Xenoestrogen-Bound ERα

ELISAs were used to measure the binding of NR-box sequences from the coactivators (A) SRC-1, (B) GRIP1, and (C) ACTR to ERα on different EREs. First, 96-well plates were coated with streptavidin and then incubated with biotinylated oligonucleotides corresponding to the vitellogenin A2, pS2, lactoferrin, or complement 3 gene EREs. PBST (no ER control) or purified ERα protein in PBST was added to each well, and a saturating concentration (2.5 μg) of GST-SRC-1 (NR-box), GST-GRIP1 (NR-box), or GST-ACTR (NR-box) fusion protein was added to individual wells and incubated with the ER target. The assays were performed in presence of vehicle (veh) or 10^{-5} M E2, Gen, Coum, Zear, BPA, NP, 3-TCB, or 4-CB. The wells were washed with PBST to remove nonbinding GST-coactivator fusion protein and incubated with an anti-GST-HRP-conjugated antibody. Immunocomplexes were detected with ABTS supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 415 nm. Each data point is the average of duplicate determinations from a representative experiment and all experiments were repeated three times with very similar results. The absorbance readings for control experiments (no ER) are indicated above the control data in each graph; these values were identical to those measured in wells containing the ABTS substrate and lacking all other components of the assay.

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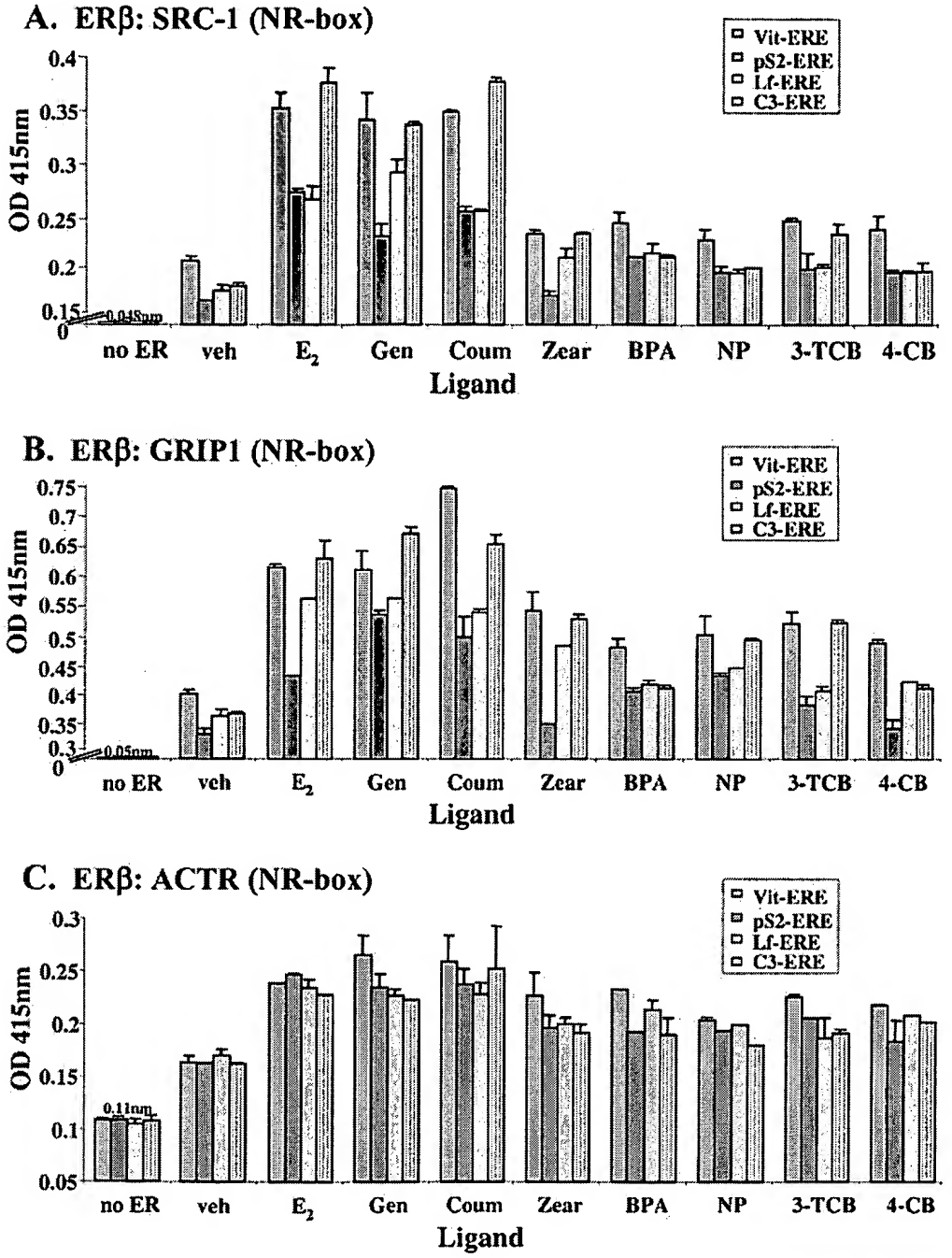

Fig. 9. The Specific ERE Sequence Influences the Recruitment of p160 Coactivators to the E2 and Xenestrogen-Bound ERβ

ELISAs were used to measure the binding of NR-box sequences from the coactivators (A) SRC-1, (B) GRIP1, and (C) ACTR to ERβ on different EREs. First, 96-well plates were coated with streptavidin and then incubated with biotinylated oligonucleotides corresponding to the vitellogenin A2, pS2, lactoferrin, or complement 3 gene EREs. PBST (no ER control) or purified ERα protein in PBST was added to each well, and a saturating concentration (2.5 μg) of GST-SRC-1 (NR-box), GST-GRIP1 (NR-box), or GST-ACTR (NR-box) fusion protein was added to individual wells and incubated with the ER target. The assays were performed in presence of vehicle (veh) or 10^{-5} M E2, Gen, Coum, Zear, BPA, NP, 3-TCB, or 4-CB. The wells were washed with PBST to remove nonbinding GST-coactivator fusion protein and incubated with an HRP-conjugated anti-GST antibody. Immunocomplexes were detected with ABTS supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 415 nm. Each data point is the average of duplicate determinations from a representative experiment and all experiments were repeated two or three times with very similar results. The absorbance readings for control experiments (no ER) are indicated above the control data in each graph; these values were identical to those measured in wells containing the ABTS substrate and lacking all other components of the assay.

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tifs were detected with ERα and ERβ, indicating that different response elements induce distinct allosteric changes in the two receptors. This finding complements recent reports describing ERE-specific differences in ERα sensitivity to protease digestion, suggesting that global changes in ER structure are induced by DNA binding (29, 31, 32). Circular dichroism spectroscopy analysis of ER-DNA interactions has also revealed that the conformation and thermal stability of ERα is altered in the ERE-bound receptor (44). Interestingly, the E2-ER-ERE complex is more stable than the E2-ER or ER-ERE alone, suggesting a combinatorial effect of ligand and DNA. Our studies also reveal that the relative ER transcriptional activities and receptor conformations on different EREs are ligand specific. Thus, ligand and response element function in concert to alter receptor structure and function, providing an explanation for the combinatorial effect of ligand and ERE context on ER transcriptional response.

An advantage of the peptide affinity approach to analyze ER structure was that we were able to specifically examine the conformation of the coactivator-binding pocket (AF-2), whereas previous studies have focused on global structural changes induced by DNA binding (29, 31, 32). The use of phage expressing different LXXLL motifs as conformational probes permitted us to determine that the structure of the ER coactivator-binding pocket is distinct on different EREs, suggesting that the response element could regulate cofactor binding. As suggested by the phage analysis, the consequences of the DNA-specific alterations in AF-2 were revealed by the observation that the binding efficacies of ERα and ERβ for the coactivators SRC-1, GRIP1, and ACTR NR-boxes were not the same on different EREs. Thus, different DNA response elements induce distinct conformations in the ER coactivator pocket, leading to differential cofactor recruitment and gene response. Interestingly, regulation of structure and function by specific DNA response elements is an emerging paradigm in nuclear receptor signaling. Significant conformational differences in the GR structure are apparent on different negative GREs, an activity that influences the efficacy of GR-mediated repression (45). The possible functional consequences of these structural alterations was demonstrated by Yamamoto *et al.* (46), showing that GR and TR form different complexes with cellular proteins when the receptors are bound to different response elements. Thus, as recently proposed by Lefstin and Yamamoto (30), response elements communicate important information to transcription factors and in this way, akin to ligand, are key regulatory components of nuclear receptor structure and function.

Differential Regulation of ERα and ERβ Structure and Function by Variant EREs

We are interested in defining the roles of ERα and ERβ in mediating cellular and physiological responses to ER ligands. Previously, we have shown that ERα and ERβ are not functionally equivalent, and each receptor plays a different role in ER action *in vitro* and *in vivo* (9–11, 22). In the current study, although saturating concentrations of xenoestrogen ligands for ERα and ERβ were used in the transactivation analyses, the relative transcriptional responses of the two receptors were distinct for each compound examined. As shown previously (47, 48), xenoestrogen-bound ERα and ERβ also differed in their coactivator preferences, further indicating that the two receptors operate through unique mechanisms.

One of the most important distinctions between ERα and ERβ, however, was that for each ligand and coactivator studied, the magnitude of transcriptional response and cofactor association differed between the two ER subtypes on each ERE examined and when compared between different response elements. While the DNA binding domains of the two receptors are virtually identical (4–6) and display similar affinities for the imperfect EREs analyzed (Ref. 26 and our unpublished observations), it appears that the ERE-bound ERα and ERβ interpret the information encoded by the specific ERE sequence in unique manners. In support of this hypothesis, using LXXLL-containing peptide probes, which bound with similar efficacy to ERα and ERβ in the absence of DNA, we found that the conformational changes elicited in the coactivator-binding pockets of the two receptors by each ERE sequence were distinct. We propose that these differences may contribute to the promoter-specific activities of the two ER subtypes observed, and furthermore, could provide a mechanism for differential gene expression by ERα and ERβ through the classical ERE pathway.

Correlation between Coactivator Recruitment on Different EREs and Transcriptional Response

One of the most important findings of this study was that ERα and ERβ transcriptional activity on ERE-containing promoters often correlated with AF-2 cofactor recruitment on the corresponding ERE sequences. Specifically, in many instances there was a direct relationship between the efficacy of coactivator binding and magnitude of transcriptional response on different response elements. Interestingly, this correlation was more apparent with ERβ, perhaps suggesting that ERβ depends more heavily on AF-2 coactivators for ligand-mediated activity through the classical ERE-mediated pathway. In support of this hypothesis, human ERβ lacks a functional AF-1 domain and thus depends solely on AF-2 for transcriptional responses (7–9). On the other hand, ERα possesses a strong AF-1 domain that binds both AF-1 and AF-2 coactivators and makes important contributions to the transcriptional activity of the receptor (24, 49–52).

The inherent properties of the ER activation domains may also explain the observation that in some instances there was no correlation between degree of

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ER-coactivator association and gene response. For example, it was surprising to discover that ERα displayed highest activity on the C3-ERE for all ligands examined even though there was no clear preference for coactivator recruitment on this element. In contrast, the relative activity of ERβ on the C3-ERE compared with other response elements closely mirrored the level of SRC-1 and GRIP1 binding. A likely explanation is that for ERα, AF-1 is the dominant activation function on the C3 promoter in HepG2 cells, suggesting an important role for AF-1 coactivators in C3 gene activation by ERα (9, 24, 53). Conversely, with ERβ, which lacks a functional AF-1, it is reasonable that the binding of p160/AF-2 coactivators more closely paralleled transcriptional activity on the C3-ERE and other response elements. Because we elected to focus on AF-2 coactivators in the current study, it is anticipated that future analyses of the influence of ERE sequences on the binding of both AF-1 and AF-2 coactivators to ERα may reveal additional correlations between activity and cofactor binding. Furthermore, transactivation studies in HeLa cells (where AF-1 has little activity) show lower activity of ERα on the C3-ERE that is more correlative with p160 coactivator binding efficacy (our unpublished data), indicating that cell context also influences the ability of EREs to regulate ER activity and coactivator recruitment.

An alternate explanation for discrepancy between ER transactivation and coactivator binding is that in some circumstances ER may bind to some cofactors in a manner that is not transcriptionally productive; thus, relative affinity would not dictate activity. For example, ACTR is known to interact with both ERα and ERβ; however, the coactivator function is selective for ERα (54). Furthermore, significant levels of coactivator binding to the unliganded ERβ were detected in the ELISAs, even though the human ERβ subtype does not display detectable ligand-independent transcriptional activity on ERE-containing promoters (9). Our results also suggest that the specific ERE sequence may influence the efficacy of ER-associated coactivators. According to this model, information encoded by the DNA sequence could be transmitted to ER-associated coregulators through protein-protein contacts with the ERE-bound receptor. Recently, it was reported that the GRIP1 homolog TIF-2 (transcriptional intermediary protein-2) functions as an activator or repressor of GR depending on the GRE context (55). Thus, it is likely that information encoded in DNA response elements is communicated to multiple components of steroid receptor transcriptional complexes, enhancing or decreasing their functionality.

In summary, this series of studies has revealed that EREs transmit information to ER by inducing alterations in receptor conformation, which in turn influence coactivator recruitment and ligand-mediated transcriptional response. It is clear that there exists a complex relationship between ER and the 3 classes of binding partners (ERE, ligand, coregulator). The observation that totality of ER transcriptional response is not always the sum of the contributions from different classes of effectors suggests that each component contributes information that influences the impact of the other two. This mechanism provides for collaborative control of gene expression by the ER-bound ligand, coregulators, and specific ERE sequence and lends additional diversity to hormone-dependent gene response. Given the high degree of structural and functional conservation among nuclear hormone receptors, it is anticipated that DNA-specific alterations in receptor structure and coregulator recruitment will serve as a universal regulatory component of hormone-responsive gene expression.

MATERIALS AND METHODS

Biochemicals

DNA restriction and modification enzymes were obtained from New England Biolabs, Inc. (Beverly, MA) or Promega Corp. (Madison, WI). PCR reagents were obtained from Perkin-Elmer Corp. (Norwalk, CT) or Promega Corp. E2, genistein, and zearalenone were purchased from Sigma (St. Louis, MO). Coum was obtained from Acros Organics (Morris Plains, NJ). Bisphenol A was purchased from Aldrich (Milwaukee, WI). Nonylphenol was purchased from Schenectady International (Schenectady, NY). 4-Hydroxy-2′,4′,6′-trichlorobiphenyl and 4-hydroxy-2′,3′,4′,5′-tetrachlorobiphenyl were purchased from Ultra Scientific (North Kingstown, RI). The horseradish peroxidase-conjugated anti-M13 antibody was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The horseradish peroxidase-conjugated anti-GST antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Streptavidin and ABTS (2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) were purchased from Sigma. Baculovirus-expressed human ERα and ERβ were purchased from PanVera Corp. (Madison, WI).

Phage ELISA

Phage ELISAs were performed as described previously with modifications (20). Ninety-six-well Immulon 4 plates (Dynex Technologies, Inc., Chantilly, VA) were coated with streptavidin (1 μg in 100 μl of 100 mM $NaHCO_3$ pH 8.5 per well) and incubated overnight at 4 C. The wells were blocked with 150 μl of 0.1% BSA in $NaHCO_3$ for 1 h at room temperature and then washed five times with PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.3], 0.1% Tween 20). Two picomoles of biotinylated Vit-, pS2- or C3-ERE oligonucleotides were added to each well and incubated for 1 h at room temperature. Saturating concentrations of purified ERα or ERβ protein for each response element (4 pmol in 100 μl PBST, as determined by ER-ERE ELISAs) were added to individual wells and incubated with the target DNA. Wells were washed with PBST and blocked a second time with 150 μl of 0.1% BSA in $NaHCO_3$ for 1 h at room temperature and then washed with PBST. Fifty microliters of 10^6 pfu titered stock for each purified phage clone (a concentration predetermined to yield maximal ER binding) was added to individual wells and incubated with the ER target for 1 h at room temperature. The assays were performed in the absence and presence of 10^{-5} M of various ER ligands. The wells were washed five times with PBST to remove nonbinding phage. A horseradish peroxidase-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted 1:5000 in PBST, 100 μl of the mixture were added to each well, and the solutions were incubated for 1 h at room temperature. The wells were

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washed five times with PBST, and immunocomplexes were detected with ABTS (2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) supplemented with 0.05% H_2O_2. The colorimetric change was quantitated by measuring the absorbance at 405 nm on a plate reader (Multiskan MS; Labsystems, Marlboro, MA).

Coactivator ELISA

The interaction of ERα and ERβ with the coactivators SRC-1, GRIP1, and ACTR on different EREs was measured using a modified version of the phage ELISA described above. Briefly, 96-well plates were coated with streptavidin overnight, blocked with 0.1% BSA in $NaHCO_3$ and then incubated with 2 pmol biotinylated oligonucleotides. A saturating concentration of purified ERα or ERβ protein (4 pmol for the Vit, pS2 and Lf-EREs; 8 pmol for the C3-ERE, as determined by ER-ERE ELISAs; see *Results*) was added to individual wells and incubated with the target DNA. Wells were washed with PBST and blocked a second time with 150 μl of 0.1% BSA in $NaHCO_3$ for 1 h at room temperature and then washed with PBST. Saturating concentrations (2.5 μg/well) of *Escherichia coli* purified GST-SRC-1 (NR-box), GST-GRIP1 (NR-box), or GST-ACTR (NR-box) fusion proteins for the assay in 100 μl PBST were added to each well, together with vehicle or 10^{-5} M of various ER ligands and incubated for 1 h at room temperature. Wells were washed ten times with PBST to remove nonbinding GST-coactivator fusion proteins. A horseradish peroxidase-conjugated anti-GST antibody was diluted 1:100 in PBST, 100 μl of the mixture were added to each well, and the solutions were incubated for 1 h at room temperature. The wells were washed eight times with PBST, and immunocomplexes were detected with ABTS (Sigma). The colorimetric change was quantitated by measuring the absorbance at 415 nm on a plate reader (Microplate Reader 550, Bio-Rad Laboratories, Inc., Hercules, CA). Saturating concentrations (2.5 μg/well) of *Escherichia coli* purified GST-SRC-1 (NR-box), GST-GRIP1 (NR-box), or GST-ACTR (NR-box) fusion proteins used in the assay were determined by titration of increasing concentrations (ranging from 0–6 μg/well) of each coactivator-fusion protein and calculating the amount yielding maximal binding in the assay by ELISA.

ERE Oligonucleotides for ELISAs

The Vit-ERE, pS2 (pS2-ERE), Lf-ERE, and C3-ERE gene EREs used in the ELISAs were generated by annealing biotinylated oligonucleotides (Life Technologies, Inc., Rockville, MD) containing the ERE sequences from the four respective estrogen-responsive genes. The sequences of the oligonucleotides used were: Vit-ERE, 5′-Biotin-gatctaggtcacagtgacctgcg and 3′-Biotin-gatccgcaggtcactgtgaccta; pS2-ERE, 5′-Biotin-gatctaggtcacggtggcctgcg and 3′-Biotin-gatccgcaggccaccgtgaccta; Lf-ERE, 5′-Biotin-gatctaggtcaaggtaacctgcg and 3′-Biotin-gatccgcaggttaccttgaccta; C3-ERE, 5′-Biotin-gatctaggtggccctgacctgcg and 3′-Biotin-gatccgcaggtcagggccaccta.

Plasmids

The mammalian expression plasmids for ERα (pcDNA-hERα) and ERβ (pcDNA-ERβ) contain the cDNAs of the human ERα and ERβ subcloned into pcDNA3 (Invitrogen, San Diego, CA). The reporters Vit-ERE-TATA-Luc, pS2-ERE-TATA-Luc, Lf-ERE-TATA-Luc, and C3-ERE-TATA-Luc were created as follows: the pGL2-TATA-Inr plasmid (gift from Dr. X. F. Wang, Duke University Medical Center, Durham, NC), containing a minimal TATA promoter upstream of a luciferase reporter gene, was digested with *Bgl*II. Overlapping oligonucleotides containing one copy of either the vitellogenin (Vit), pS2, lactoferrin (Lf), or complement 3 (C3) ERE and *Bgl*II sites on the ends were annealed and ligated into pGL2-TATA-Inr. The sequences of the oligonucleotides used were: Vit 5′-gatctaggtcacagtgacctgcg and 3′-gatccgcaggtcactgtgaccta, pS2 5′-gatctaggtcacggtggcctgcg and 3′-gatccgcaggccaccgtgaccta, Lf 5′-gatctaggtcaaggtaacctgcg and 3′-gatccgcaggttaccttgaccta, and C3 5′-gatctaggtggccctgacctgcg and 3′-gatccgcaggtcagggccaccta. All constructs were sequenced to verify the presence of 1 copy of ERE oligonucleotide in the correct orientation.

The pGEX-SRC-1 (NR), pGEX-GRIP1 (NR), and pGEX-ACTR (NR) plasmids contain the nuclear receptor interacting domains (including the 3 LXXLL motifs) from the coactivators SRC-1, GRIP1, and ACTR fused to the GST tag in the pGEX-5X-1 parent plasmid (Amersham Pharmacia Biotech). pGEX-SRC-1 (NR) (22) contains sequence coding for amino acids 621–765 of SRC-1; pGEX-GRIP1 (NR) (gift from Dr. M. Stallcup, University of Southern California, Los Angeles, CA) contains sequences for amino acids 730-1121 of GRIP1 (56). pGEX-ACTR (NR) (gift from Dr. X. Li, Duke University Medical Center) contains sequences encoding amino acids 620–820 of ACTR.

Cell Culture and Transient Transfection Assays

HepG2 cells were maintained in minimum essential medium (Life Technologies, Inc.) supplemented with 10% FCS, 2 mM glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Inc.). Cells were plated in 24-well plates (coated with 0.1% gelatin) 24 h before transfection. DNA was introduced into the cells using Fugene 6 (Roche Diagnostics Corp., Indianapolis, IN). In standard transfections, 500 ng of reporter (Vit-ERE-TATA-Luc, pS2-ERE-TATA-Luc, Lf-ERE-TATA-Luc or C3-ERE-TATA-Luc), 90 ng of receptor (either pcDNA-hERα or pcDNA-hERβ), and 10 ng of the pRL-CMV renilla luciferase normalization vector (Promega Corp.) were used for each well, and all transfections were performed in triplicate. Before transfection, cells were washed with PBS, and 200 μl phenol red-free MEM containing 10% charcoal-stripped FCS (HyClone Laboratories, Inc., Logan, UT) was added to each well. Cells were incubated with the DNA/fugene mix for 6 h and then receptor ligands in 200 μl phenol red-free MEM were added to the cells and incubated for 20 h. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocols (Promega Corp.). Each value was normalized to the renilla luciferase control, and each data point generated is the average of triplicate determinations. All experiments were repeated three times.

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